


# Elevated Ratio of Maternal Plasma ApoCIII to ApoCII in Preeclampsia

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## Abstract

Preeclampsia is a hypertensive disorder unique to pregnancy. Although the pathogenesis of the disease begins with aberrant spiral artery invasion in the first trimester, clinical symptoms usually do not present until late in pregnancy. Apolipoprotein CII (ApoCII) and its negative regulator, apolipoprotein CIII (ApoCIII), have recently been described as atherogenesis biomarkers in models of cardiovascular disease. Given the similarities in pathology, etiology, and clinical presentation between cardiovascular disease and preeclampsia, we hypothesized that the ratio of ApoCIII to ApoCII in maternal first trimester plasma would predict preeclampsia later in pregnancy. To test this hypothesis, plasma was prospectively collected from 311 nulliparas at 8 to 12 weeks gestation. After delivery, patients were divided into cohorts based on preeclampsia diagnosis. Conditioning monocytes with preeclamptic plasma potentiated monocyte adhesion to endothelial cells in an in vitro model. The ratio of ApoCIII to ApoCII was significantly elevated in patients with severe preeclampsia relative to normotensive and gestational hypertensive individuals ( $P < .05$ ) as determined by mass spectrometry and competitive enzyme-linked immunosorbent assay (ELISA) assays. These results support a predictive change in the ratio of ApoCIII to ApoCII in pregnancies complicated by severe preeclampsia.

## Keywords

apolipoprotein C, preeclampsia, triglyceride, glycosylation

## Introduction

Preeclampsia is a pregnancy-specific disorder affecting approximately 5% to 7% of nulliparous pregnancies and is one of the leading causes of maternal and neonatal morbidity and mortality nationwide.<sup>1</sup> Preeclampsia initiates in the first trimester with incomplete trophoblast invasion of maternal spiral arteries. The multiorgan maternal syndrome is not typically diagnosed until the third trimester.<sup>2</sup> Aberrant placental perfusion initiates an exaggerated inflammatory response, elevated plasma triglycerides, and endothelial cell damage. These factors predispose the patients to generalized vasoconstriction, resulting in proteinuria and hypertension later in gestation.<sup>2</sup>

The presentation of preeclampsia varies significantly between individuals.<sup>3</sup> Although both dyslipidemia and endothelial dysfunction are characteristic of preeclampsia,<sup>3,4</sup> a mechanism unifying the 2 phenomena remains to be established. Dyslipidemia becomes apparent in most patients with preeclampsia before clinical presentation (ie, in the first and second trimester).<sup>3</sup> Lipid-laden macrophages are found in the spiral arteries of patients with preeclampsia. However, the etiology of these macrophages is currently unknown, and their relevance to the clinical manifestations of the disease is controversial.<sup>5</sup> In vitro models of endothelial dysfunction in cardiovascular disease link the triglyceride-associated plasma

protein apolipoprotein CIII (ApoCIII) with monocyte adhesion to endothelial cells.<sup>6</sup> Apolipoprotein CIII functions primarily as an inhibitor of the tissue lipoprotein lipase cofactor ApoCII.<sup>7</sup> Current literature speculates that ApoCIII plasma concentration and the ratio of ApoCIII to ApoCII may link the immunodysfunction and dyslipidemia components, leading to atherogenesis.<sup>8,9</sup> We hypothesize that a similar mechanism may be applicable to the atherosclerosis of preeclampsia.

Apolipoprotein CIII elevates plasma triglyceride levels by preventing plasma clearance of VLDLs, HDLs, and chylomicron remnants. Different ApoCIII glycoisoforms in plasma are codified by the number of associated sialyl residues: ApoCIII<sub>0</sub>, ApoCIII<sub>1</sub>, and ApoCIII<sub>2</sub>. Increased sialylation has been associated with hypertriglyceridemia.<sup>10</sup> The elevated ratio of ApoCIII<sub>1</sub> to ApoCIII<sub>2</sub> is a risk factor for several disease states

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**Table 1.** Patient Demographics, Obstetric Outcomes, and Laboratory Values by Cohort<sup>a</sup>

	Maternal Age at Intake	Gestational Age at Delivery (weeks)	Prepregnancy Body Mass Index	Hemoglobin at Intake	Hematocrit at Intake	Baseline Cholesterol (mg/dL)
Normotensive	24 ± 5	39 ± 1	23 ± 3	13 ± 1	38 ± 3	174 ± 39
Gestational hypertensive	25 ± 5	39 ± 1	26 ± 4	14 ± 1	40 ± 2	208 ± 75
Mild preeclampsia	23 ± 3	38 ± 1	26 ± 4	13 ± 1	39 ± 3	187 ± 50
Severe preeclampsia	25 ± 6	32 ± 5 <sup>b</sup>	26 ± 4	14 ± 1	39 ± 3	177 ± 37

<sup>a</sup> Values represent mean ± SD.

<sup>b</sup>  $P < .016$  relative to normotensive cohort by Mann-Whitney test with Bonferroni correction.

associated with hypertriglyceridemia, including liver disease, graft versus host disease, and obesity.<sup>11</sup> Apolipoprotein and serum lipid levels increase exponentially during gestation as part of the well-characterized hypertriglyceridemia of pregnancy.<sup>12</sup> However, data on the correlation between elevated triglycerides and apolipoproteins and the onset of preeclampsia are conflicting. Many studies reporting no correlation are limited by the sensitivity of the technology used to identify and quantify apolipoprotein levels.<sup>11,13-15</sup>

The objective of our study was to use a mass spectrometry-based assay to determine whether the ratio of ApoCIII to ApoCII is modulated in the first trimester of pregnancies complicated by preeclampsia relative to normotensive controls and patients with gestational hypertension. We hypothesize that the ratio of ApoCIII to ApoCII will be significantly elevated in preeclamptic plasma relative to the remaining cohorts in the first trimester, well before onset of clinical symptoms.

## Patients and Methods

### Participants

Healthy, nulliparous women aged 18 years or older without a history of chronic medical conditions (eg, preexisting diabetes mellitus, neurological disorders, cardiovascular anomalies, etc) or infertility treatment were approached to participate in the study during their initial intake appointment at 8 to 12 weeks gestation by first day of the last menstrual period (LMP). A total of 311 patients gave informed consent per the Madigan Healthcare System (Madigan) Institutional Review Board approval of the study protocol. Investigators adhered to the policies for protection of human participants per Madigan regulations. Blood was collected by venipuncture in EDTA Vacutainer tubes (Beckman-Dickinson, Franklin Lakes, New Jersey) at 8 to 12 weeks' gestation. Per standard obstetric care, vitals including body mass index (BMI) and blood pressure were measured at initial and all subsequent obstetric appointments and were available for investigator review. Medical records were screened postpartum and patients were subdivided into cohorts ( $n = 5$ -11 patients each) based on antepartum diagnosis of preeclampsia, gestational hypertension, or normotensive control.<sup>16</sup> Two patients with superimposed preeclampsia (ie, with chronic hypertension based on elevated blood pressures prior to 20 weeks' gestation) were excluded from analysis. Two patients with HELLP (hemolysis,

elevated liver enzymes, low platelets) syndrome were likewise excluded from analysis. Patients with preeclampsia were further stratified into "mild" or "severe" per diagnostic criteria provided by the American College of Obstetrics and Gynecology (ACOG) Practice Bulletin based on antepartum blood pressure and proteinuria results.<sup>16,17</sup> Patients with mild preeclampsia delivered close to term, had proteinuria ( $\geq 0.3$ g in a 24-hour urine culture), and had blood pressures of  $\geq 140$  mm Hg systolic or 90 mm Hg diastolic after 20 weeks' gestation. Severe preeclampsia was diagnosed by blood pressures greater than 160 mm Hg systolic or 110 mm Hg diastolic on 2 occasions at least 6 hours apart. Patients with severe preeclampsia had proteinuria of 5 g or higher in a 24-hour urine specimen or 3+ or greater on 2 random urine samples collected at least 4 hours apart. None of the patients included in the cohorts had conditions predisposing them to preeclampsia (ie, a history of chronic hypertension, pregestational diabetes, vascular and connective tissue disease, multifetal gestation, nephropathy, obesity [defined by BMI greater than 30 at intake appointment], or antiphospholipid syndrome). Ages ranged from 19 to 40 years (Table 1). Ethnicities were balanced among cohorts. A review of patient prescriptions confirmed that none of the patients had been prescribed fibrates, statins, or any other pharmacological agent known to modulate plasma triglyceride levels.<sup>8</sup> Patients were prescribed prenatal vitamins containing niacin as standard of care, but patients in all cohorts received comparable doses. At time of phlebotomy at 8 to 12 weeks' gestation, none of the patients in any cohort had active prescriptions for antihypertensive drugs. Patient demographics and pertinent vitals and laboratory test results are presented in Table 1. Total cholesterol was determined by enzymatically oxidizing plasma cholesterol and measuring  $H_2O_2$  by colorimetric assay according to the manufacturer's instructions (Catalog #K603-100, Bio-Vision Research Products, Mountain View, California).

### Adhesion Assays

Human umbilical cord vein endothelial cells (HUVECs, CRL-1730) and human histiocytic lymphoma monocytes (U937, CRL-1593.2) were cultured in a 96-well format according to supplier recommendations (American Tissue Culture Collection, Manassas, Virginia). Adhesion assays were performed according to published protocols.<sup>6,18</sup> Briefly, U937 monocytes were seeded at  $0.25 \times 10^6$  cells/mL in assay buffer (Hank's

balanced salt solution, pH 7.4, supplemented with 0.1% bovine serum albumin, Sigma Chemicals, St Louis, Missouri) containing 5% maternal plasma or vehicle control for 16 hours. Monocytes were washed once and resuspended in 1 mmol/L BCECF/AM (2',7'-bis-(Carboxyethyl)-5(6')-carboxyfluorescein acetoxymethyl ester; Calbiochem/EMD, San Diego, California) for 25 minutes at 37°C. Cells were washed and allowed to adhere to HUVEC monolayers for 15 minutes. Total fluorescence was determined by microplate fluorescence spectrophotometer (Biotek Instruments, Winooski, Vermont). Nonadherent cells were transferred to a clean plate. Fluorescence of both adherent and supernatant monocytes was quantified by spectrophotometer. Adherent cells were imaged at  $\times 40$  magnification with 488 nm excitation and  $\times 200$  magnification with visible light on an Olympus IX-71 reflected fluorescence microscope (Melville, New York) equipped with an inline CCD camera.

### Plasma Preparation and SELDI-TOF Mass Spectrometry

Plasma was isolated by centrifugation at 1500g for 15 minutes at 4°C in a Sorvall RC3C centrifuge (Global Medical Instrumentation, Ramsey, Minnesota). Plasma supernatants were supplemented with a combination of protease inhibitors according to manufacturer instructions (11-836-145-001, Roche Diagnostics, Mannheim, Germany) and stored at  $-140^{\circ}\text{C}$  or  $-150^{\circ}\text{C}$ . Plasma was denatured by vortexing for 20 minutes at room temperature in urea/chaps buffer (9M urea, 2% chaps in 50 mmol/L TrisHCl, pH 9) in preparation for application onto Surface-Enhanced Laser Desorption Ionization Time of Flight Mass Spectrometry (SELDI-TOF MS) hydrophobic (H50) arrays (Bio-Rad Laboratories, Hercules, California). Prior to specimen application, H50 arrays were hydrated in water:methanol, 50:50, washed in binding buffer (10:89.9:0.1 acetonitrile:water:trifluoroacetic acid), then incubated for 60 minutes at room temperature with denatured plasma diluted in binding buffer on a Micromix orbital shaker (setting 20/5; Diagnostics Products Corporation, Flanders, New Jersey). Arrays were washed to remove unbound proteins and desalted by rinsing in deionized distilled water. Sinapinnic acid (50% saturated) dissolved in acetonitrile and trifluoroacetic acid was added to each spot using a Biodot Robot (Biodot Inc, Irvine, California) and allowed to evaporate in situ. Plasma proteins were laser desorbed from the hydrophobic arrays using a PCS4000 Enterprise Edition SELDI-TOF MS analyzer (Bio-Rad). Laser intensity was determined empirically. After 2 warming shots, 10 data shots were fired, spatially sampled, and digitally analyzed over 10 passes (partitioned into 530 of 2120 evenly spaced pixels per spot). Data were preprocessed according to manufacturer specifications by subtracting baseline, reducing noise to 0.2% peak width, and normalizing spectra to total ion current to correct for minor differences in concentration among specimens, including the generalized dilution with blood volume increase in pregnancy.<sup>19</sup> Spectral

masses were externally calibrated to protein standards at 4 masses (6.963, 12.231, 16.951, and 29.023 kDa [Bio-Rad]).

### O-Linked Glycosidase Treatment

Plasma was treated overnight with O-glycosidase from *Diplococcus pneumoniae* (25 mU/50  $\mu\text{L}$ , Roche Diagnostics) or neuraminidase from *Vibrio cholera* (1 U/mL, Roche Diagnostics) in reaction buffer (200 mmol/L sodium acetate, 2 mmol/L calcium chloride, pH 4) as described.<sup>20</sup> SELDI-TOF MS analysis was performed as described above.

### Protein Purification and Tandem Mass Spectrometry

To procure an adequate quantity of material for protein purification, plasma was pooled from 19 confirmed normotensive individuals (100  $\mu\text{L}$  per patient, to include the 10 normotensive patients screened in this analysis). Plasma was purified using an established method of sequential chromatography with strong anion exchange (SAX) followed by reverse phase (C18) column purification (Bio-Rad Laboratories). Sequential elutions were collected at 5 pH ranges (pH 9, pH 7, pH 5, pH 4, and pH 3), followed by an organic wash. The pH was adjusted by sodium hydroxide addition, and specimens were run on 10% tricine gels (Invitrogen, Carlsbad, California) and stained with colloidal blue total protein stain (Invitrogen). Bands at approximately 9 kDa were excised and digested overnight with trypsin (Roche Diagnostics). An undigested aliquot was analyzed by SELDI-TOF MS to confirm recovery of spectral features in the 8 to 10 kDa mass range. Tryptic fragment masses were ascertained by QSTAR XL mass spectrometer (ABI/Sciex, Foster City, CA) equipped with a PCI-1000 ProteinChip Interface (Ciphergen Biosystems, Fremont, CA) and the resulting collision-induced dissociation (CID) spectral data were submitted to the database mining tool Mascot (Matrix Sciences, Boston, MA) or Protein Prospector MS-Tag (UCSF, The Regents of the University of California, San Francisco, CA) for identification by fragmentation pattern.

### Enzyme-Linked Immunosorbent Assay

Competitive enzyme-linked immunosorbent assays (ELISAs) were performed according to manufacturer specifications (Assay Pro, St Charles, Missouri). Monoclonal antibodies specific for ApoCII or ApoCIII had minimal cross-reactivity for other apolipoproteins.

### Statistical Evaluation

To determine statistical significance among cohorts for patient laboratory data and gestational age at delivery, (PASW, Predictive Analysis SoftWare (IBM, Armonk, New York)) software (v18) was used to conduct a Kruskal-Wallis analysis of variance (ANOVA) by ranks followed by a Mann-Whitney nonparametric multiple comparisons analysis with Bonferroni correction. Significance was set at an  $\alpha$  level of .05 (.016 with Bonferroni correction).

For analysis of mass spectrometry data, statistical significance between groups was determined by 1-way ANOVA and post hoc analysis with Bonferoni correction. When heterogeneity was found between groups, Dunnett T3 multiple comparisons analysis was used to determine group differences. In the case of smaller sample size that might not fulfill parametric assumptions, it was more appropriate to use the Kruskal-Wallis analysis for testing multiple group differences. ProteinChip Data Manager (v3.5, Bio-Rad Laboratories) was used to determine differences between groups in terms of protein peak intensities by Kruskal-Wallis analysis. All statistical tests were selected at an  $\alpha$  level of .05 (.016 with Bonferoni correction).

## Results

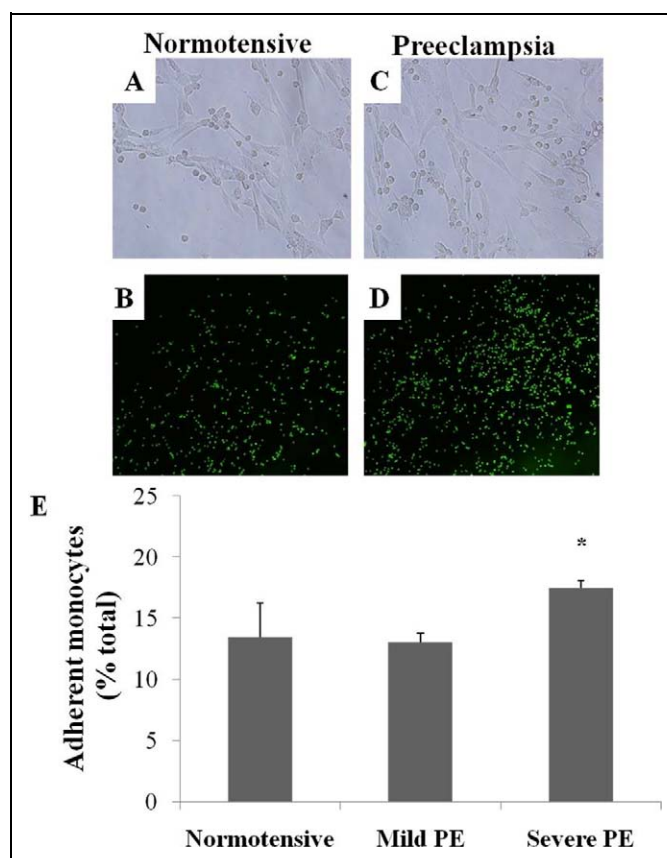
Review of antepartum records indicated that cohorts were uniformly matched by prepregnancy BMI, maternal age and ethnicity, hematocrit, and baseline cholesterol at 8 to 12 weeks' gestation (Table 1). Prematurity incidence was significantly higher in the severe preeclamptic cohort relative to the remaining cohorts (mean gestational age of delivery of  $32.2 \pm 5$  weeks,  $P = .001$ ; Table 1). Of the 311 patients enrolled in the study, we had 13 confirmed cases of preeclampsia ( $n = 8$  mild and  $n = 5$  severe) based on clinical diagnostic criteria reported in the Methods (4.2% of the study population). All other parameters, including ethnicity, were comparable among cohorts (Table 1).

### Physiological Assessment of Preeclamptic Plasma Immunoreactivity in the First Trimester

To determine whether maternal plasma was immunoreactive in the preclinical stages of preeclampsia, in vitro adhesion assays were conducted using U937 monocytes preconditioned with first trimester plasma derived from patients with confirmed diagnoses of normotensive or severe preeclampsia. First trimester plasma from patients with severe preeclampsia slightly potentiated U937 monocyte adhesion to HUVEC monolayers (Figure 1;  $17.5 \pm 0.88$  AU [arbitrary units] relative to  $13.5 \pm 0.76$  AU in normotensive and  $13.0 \pm 0.6$  AU in mild preeclampsia;  $P = .004$ ).

### SELDI-TOF MS for ApoCIII and ApoCII

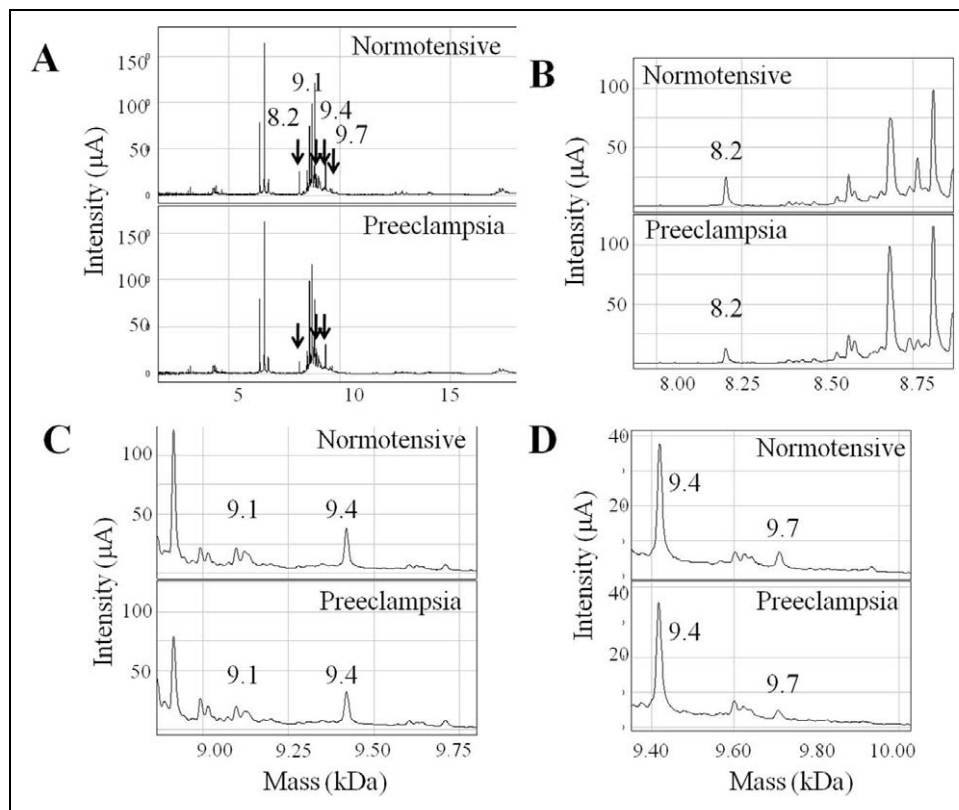
To determine whether ApoCII and ApoCIII glycoisoforms could be adequately measured in maternal plasma by SELDI-TOF MS, gestationally age-matched normotensive controls at 8 to 12 weeks' gestation were compared to plasma from patients later diagnosed with preeclampsia (Figure 2). Spectral features comigrated with proteins identified in the literature as ApoCII (8.2 kDa), ApoCIII<sub>1</sub> lacking the terminal sialic acid residue (9.1 kDa), ApoCIII<sub>1</sub> (9.4 kDa), and ApoCIII<sub>2</sub> (9.7 kDa; Figure 2A).<sup>10,11</sup> While ApoCII (8.2 kDa) and ApoCIII<sub>2</sub> (9.7 kDa) decreased in abundance in the preeclamptic cohort (Figures 2B and D, respectively), spectral features consistent



**Figure 1. Preconditioning with maternal severe preeclamptic plasma increases monocyte adhesion to endothelial monolayers.** A-D, Representative images at  $\times 200$  with visible light illustrate monocytes (round cells) adhering to endothelial monolayers (elongated cell bodies) (A,C). Representative images at  $\times 40$ , 488 nm wavelength (B,D) show an increase in the number of adherent fluorescent BCECF/AM-monocytes after monocyte preconditioning with normotensive first trimester plasma (8-12 weeks gestation) (A, B). The increase in adhesion was more pronounced in the first trimester plasma from patients who later developed severe preeclampsia (C,D). E, Data from 2 independent experiments were quantified by microplate spectrophotometer at 485 nm excitation/530 nm emission ( $n = 6$  patients per cohort, triplicate wells). \* $P < .05$  by nonparametric Mann-Whitney statistical analysis. BCECF/AM indicates (2',7'-bis-(Carboxyethyl)-5(6')-carboxyfluorescein acetoxymethyl ester).

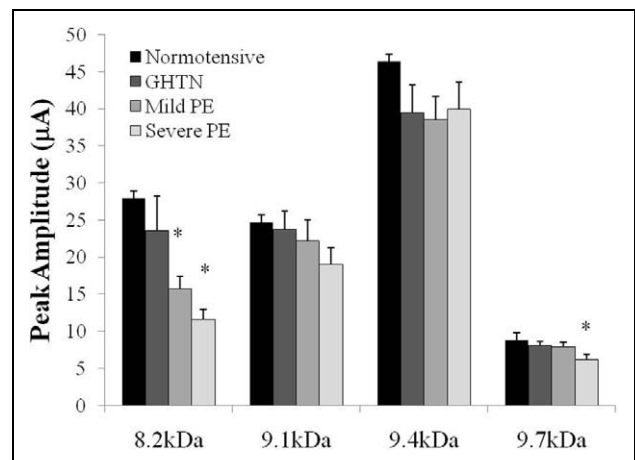
with ApoCIII<sub>1</sub> (9.4 kDa) and ApoCIII<sub>1</sub> lacking terminal sialic acid (9.1 kDa) remained unchanged (Figure 2C). Amplitude of the 8.2 kDa protein (Figure 2B) showed a greater attenuation than the 9.7 kDa protein (Figure 2D).

Gel excision of a 9 to 10 kDa fragment and subsequent tandem mass spectrometry (QTOF MS/MS) resulted in 3 peptide fragments (1.197, 1.717, and 1.907 kDa) matching the predicted sequence of ApoCIII (gi224917) and 1 peptide fragment with homology to ApoCIII<sub>0</sub> (0.898 kDa), with a homology score of 258 representing 64% sequence coverage. Further sequence analysis indicated that the protein in the 9 kDa gel fragment was glycosylated on threonine residue 74, possibly accounting for the shift in the observed mass (9.1-9.4 kDa) relative to the predicted mass (8.76 kDa).

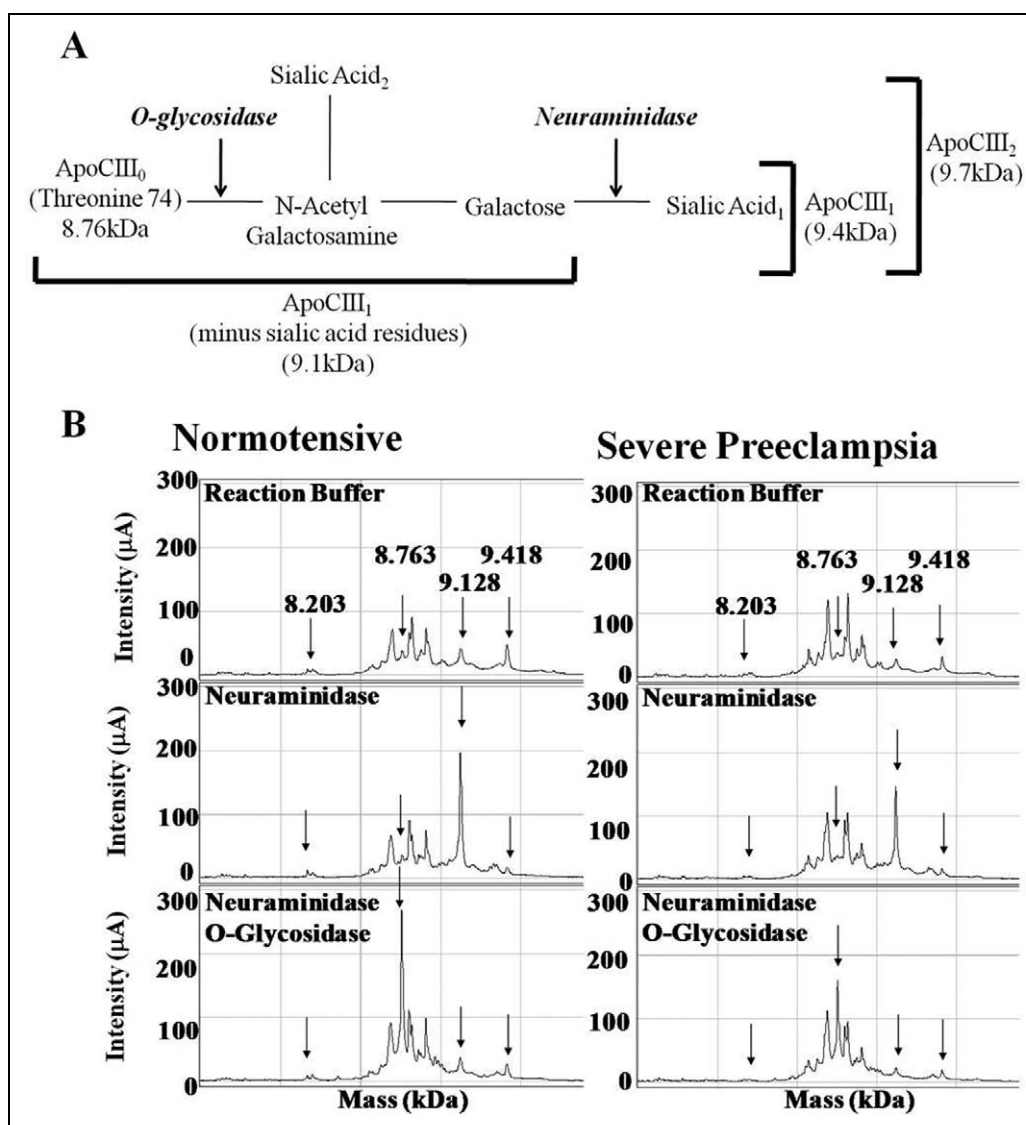


**Figure 2.** Decrease in intensity of spectral features corresponding to ApoCII and ApoCIII glycoisoforms in severe preeclampsia at 8 to 12 weeks gestation. A, Representative patients from the normotensive and severe preeclampsia cohorts at 8 to 12 weeks' gestation were profiled in triplicate by SELDI-TOF MS. Protein masses are indicated in kiloDaltons (kDa) corresponding to (B) presumptive ApoCII (8.2 kDa), (C) ApoCIII<sub>1</sub> glycoisoforms (9.1 and 9.4 kDa), and (D) ApoCIII<sub>2</sub> (9.7 kDa). ApoCII indicates apolipoprotein CII; SELDI-TOF MS, surface-enhanced laser desorption/ionization time of flight mass spectrometry.

SELDI-TOF MS optimized in the 8 to 10 kDa region of the spectrum was used to profile gestationally age-matched women (8–12 weeks gestation) in each of the 4 cohorts: normotensive, gestational hypertensive, mild preeclampsia, and severe preeclampsia ( $n = 5$ –11 per group). Considerable interindividual variability was observed among gestational hypertensives and normotensives (Figure 3). The proteins corresponding in spectral mass to ApoCIII<sub>1</sub> glycoisoforms (9.1 and 9.4 kDa) were modestly lower in abundance in the patients with severe preeclampsia relative to the other cohorts, although the difference was not statistically significant. The 8.2 kDa ApoCII protein peak was significantly attenuated in both the severe ( $11.53 \pm 2.78 \mu\text{A}$ ,  $P = .005$ ) and mild ( $15.73 \pm 3.4 \mu\text{A}$ ,  $P = .044$ ) preeclamptic plasma relative to the normotensive cohort ( $27.95 \pm 7.1 \mu\text{A}$ ). The 9.7 kDa ApoCIII<sub>2</sub> peak was significantly attenuated in the severe preeclampsia cohort ( $6.15 \pm 0.84 \mu\text{A}$ ) relative to the normotensive patients ( $8.76 \pm 0.47 \mu\text{A}$ ,  $P = .041$ ). A modest decrement was also observed in the 8.2 and 9.1 kDa proteins in patients with severe preeclampsia relative to patients with mild preeclampsia, although the difference was not statistically significant (Figure 3). Prematurity is a possible confounding variable in the patients with severe preeclampsia. Therefore, we plotted gestational age at delivery by amplitude of the 8.2 and 9.7 kDa peaks. Correlation coefficients did not suggest that



**Figure 3.** Differential ApoCII and ApoCIII<sub>2</sub> expression in preeclampsia relative to normotensive controls. Maternal plasma from gestational hypertensives ( $n = 10$ ), mild preeclamptics ( $n = 8$ ), severe preeclamptics ( $n = 5$ ), or gestationally age-matched normotensives (8–12 weeks,  $n = 11$ ) was profiled by SELDI-TOF MS in replicates of 5 to 6. Peak amplitudes were measured and compared among cohorts. \* $P < .05$ , by ANOVA and the Dunnett T3 multiple comparisons test in heterogeneous variances. ApoCII indicates apolipoprotein CII; SELDI-TOF MS, surface-enhanced laser desorption/ionization time of flight mass spectrometry; ANOVA, analysis of variance.



**Figure 4.** Apolipoprotein CIII<sub>1</sub> glycoisoforms are reduced in severe preeclamptic plasma relative to normotensive controls at 8 to 12 weeks' gestation. **A**, Chemistry of O-glycosidase and neuraminidase reactions in ApoCIII<sub>0</sub>, ApoCIII<sub>1</sub>, and ApoCIII<sub>2</sub> glycoisoforms. **B**, Maternal plasma ( $n = 4$  normotensive or severe preeclampsia patients at 8 to 12 weeks' gestation) was treated overnight with reaction buffer (upper panels, all glycovariants present), neuraminidase (middle panels, removes sialic acid residue from ApoCIII<sub>1</sub> at 9.4 kDa to create a single peak at 9.1 kDa), or neuraminidase and O-glycosidase enzymes combined (lower panels, ApoCIII<sub>0</sub>, deglycosylated, at 8.76 kDa). Treated plasma was profiled by SELDI-TOF MS. Arrows indicate ApoCIII<sub>1</sub> (9.4 kDa), ApoCIII<sub>1</sub> minus sialic acid (9.1 kDa), deglycosylated ApoCIII<sub>0</sub> (8.76 kDa), and ApoCII (8.2 kDa). The 8.2 kDa ApoCII peak was unaffected in amplitude or mass by either enzyme. ApoCIII indicates apolipoprotein CIII; SELDI-TOF MS, surface-enhanced laser desorption ionization time of flight mass spectrometry.

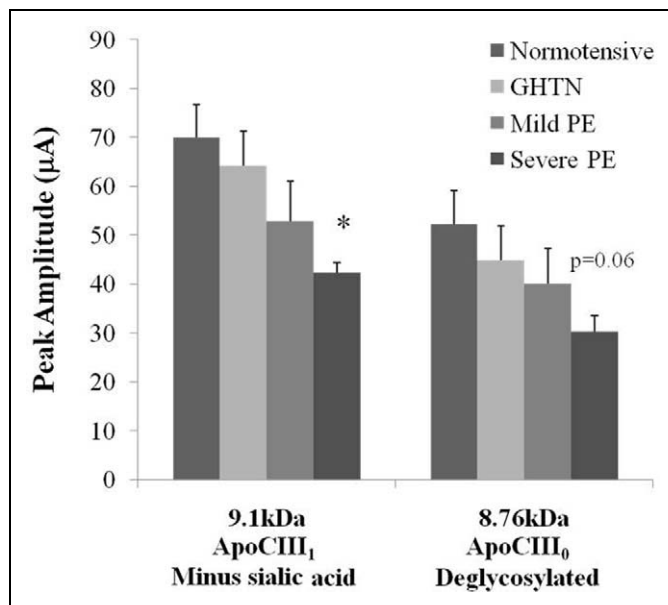
either peak amplitude varied appreciably with gestational age at delivery ( $R^2 = 0.3-0.5$ ).

To verify that the spectral features identified by SELDI-TOF MS in our study were consistent with glycoisoforms of ApoCIII reported in the literature,<sup>10,11</sup> we incubated maternal plasma with enzymes catalyzing hydrolysis of O-linked glycosylation sites (Figure 4A). Neuraminidase (sialidase) treatment resulted in the elimination of the 9.4 kDa peak (Figure 4B, middle panels) and concomitant emergence of a peak at 9.1 kDa (Figure 4B, middle panels). Incubation with both neuraminidase and O-glycosidase collapsed the 9.1 and 9.4 kDa peaks into a single peak at 8.76 kDa (Figure 4B, lower panels). These

results are consistent with 2 glycoisoforms of a protein at 8.76 kDa, a mass coincident with the unglycosylated form of ApoC-III.<sup>11,21</sup> The relative abundance of the spectral feature at 8.2 kDa was unaffected by O-linked glycosidase treatment (see Figure 4B, all panels). The literature reports that ApoCII lacks the serine/threonine sites for O-linked glycosylation and migrates at 8.2 kDa, consistent with the spectral features observed in Figure 4B.

To determine whether preeclampsia predisposed individuals to lower levels of total ApoCIII<sub>0</sub> irrespective of glycosylation state, the sialic acid and N-acetyl galactosamine/galactose residues were removed by sequential enzymatic hydrolysis.





**Figure 5.** Apolipoprotein CIII is lower in abundance in maternal plasma derived from patients with severe preeclampsia than normotensive controls and patients with gestational hypertension. Neuraminidase treatment of maternal plasma from normotensive, gestational hypertensive, or mild or severe preeclampsia cohorts resulted in a significant decrease in ApoCIII<sub>1</sub>, desialyated (left panel) in the severe preeclampsia cohort. Deglycosylated ApoCIII<sub>0</sub> resulting from treatment with both neuraminidase and O-glycosidase was modestly but not significantly decreased in the severe preeclampsia cohort (right panel). \* $P < .05$  by Kruskal Wallis ANOVA by ranks. ApoCIII indicates apolipoprotein CIII; ANOVA, analysis of variance.

Glycosylated ApoCIII<sub>1</sub> at 9.4 kDa and ApoCIII<sub>2</sub> at 9.7 kDa were collapsed into a single peak at 9.1 kDa by neuraminidase treatment to remove the terminal sialic acid residue (Figure 4B, middle panels, and Figure 5). The resulting peak amplitude was significantly different in the patients with severe preeclampsia ( $69.9 \pm 13.6 \mu\text{A}$  in the normotensives relative to  $42.3 \pm 4.2 \mu\text{A}$  in the severe preeclampsia cohort; Figure 4, middle panels, and Figure 5, left panel;  $P = .03$ ). The 9.4 and 9.1 kDa peaks were further collapsed into a single peak at 8.76 kDa, representing fully deglycosylated ApoCIII<sub>0</sub> by treatment with neuraminidase and O-glycosidase (Figure 4B, lower panels). Although the peak amplitude was reduced in the patients with severe preeclampsia relative to normotensive controls and gestational hypertensives, the decrement was not statistically significant ( $52.3 \pm 13.8$  in the normotensive individuals relative to  $30.3 \pm 6.3 \mu\text{A}$  in the patients with severe preeclampsia; Figure 5, right panel;  $P = .06$ ).

The ratios of ApoCIII<sub>1</sub> to ApoCII and ApoCIII<sub>2</sub> to ApoCII were significantly potentiated in patients with severe preeclampsia relative to normotensive controls (Figure 6, left and right panels, respectively). Ratios for mild preeclampsia were not statistically different from gestational hypertensive and normotensive patients (Figure 6). The ratio of ApoCIII<sub>1</sub> to ApoCII (9.4:8.2 kDa) were  $2.01 \pm 0.31$ ,  $2.05 \pm 0.27$ , and  $2.72 \pm 0.36$  for the normotensive, gestational hypertensive,

and mild preeclampsia cohorts relative to  $3.85 \pm 0.90$  in the patients with severe preeclampsia ( $P = .018$  by ANOVA with post hoc analysis by Bonferoni correction). The ratio of ApoCIII<sub>2</sub> to ApoCII (9.7:8.2 kDa) were  $0.182 \pm 0.03$ ,  $0.208 \pm 0.03$ , and  $0.295 \pm 0.04$  in normotensive, gestational hypertensive, and mild preeclampsia cohorts, respectively, relative to patients with severe preeclampsia ( $0.326 \pm 1.04$ ;  $P = .024$  by ANOVA and post hoc analysis with Bonferoni correction).

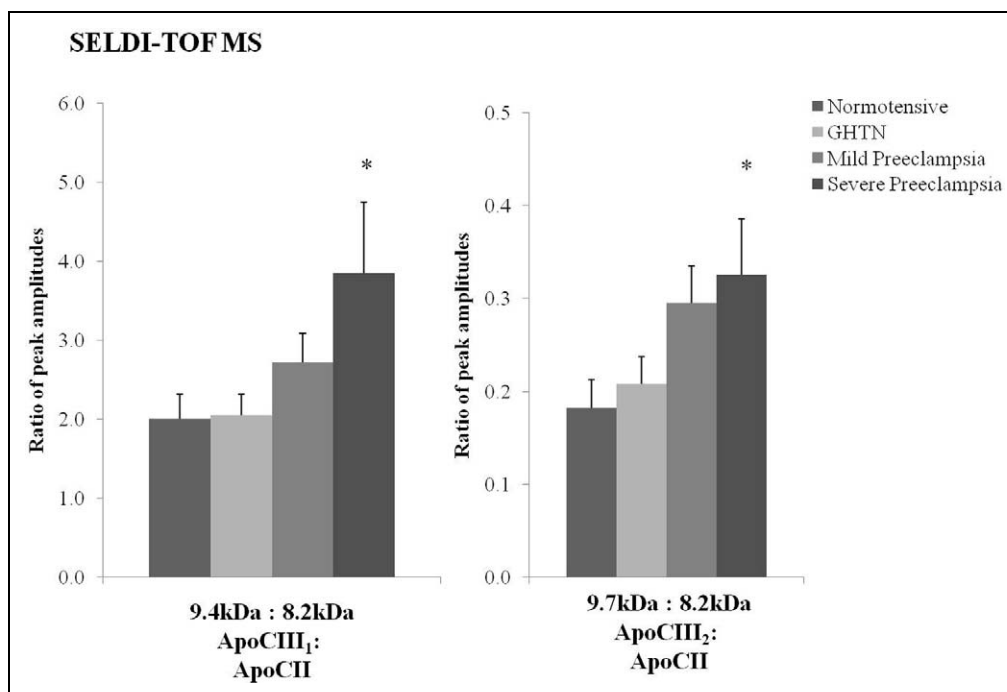
Because SELDI-TOF MS detects only qualitative changes in protein abundance, we confirmed our mass spectrometry results by quantitative competitive ELISA. The ratio of ApoCIII to ApoCII was quantifiably elevated in patients with severe preeclampsia relative to normotensive controls and mild preeclampsia cases (Figure 7;  $5.13 \pm 1.04$  in severe preeclampsia relative to  $1.29 \pm 0.22$  and  $1.99 \pm 0.18$  in normotensives and mild preeclampsia, respectively;  $P = .002$ ). Because plasma ApoCII and ApoCIII levels in gestational hypertensive patients were consistently indistinguishable from normotensive controls in the SELDI-TOF MS experiments, ApoC was not quantified by ELISA in the gestational hypertensive cohort. Similar to the SELDI-TOF MS conclusions, quantitative ELISA data indicated that the decrement in ApoCII observed in patients with severe preeclampsia is more pronounced than ApoCIII. Apolipoprotein CII mean plasma concentrations were  $163.3 \pm 21.8$ ,  $100 \pm 10.4$ , and  $32.0 \pm 0.88 \mu\text{g/mL}$  in normotensive, mild preeclampsia, and severe preeclampsia cohorts, respectively. Apolipoprotein CIII mean plasma concentrations were  $189.9 \pm 30.8$ ,  $186 \pm 19.0$ , and  $165.8 \pm 24.3 \mu\text{g/mL}$  in normotensive, mild preeclampsia, and severe preeclampsia cohorts, respectively.

## Discussion

In our population of nulliparous patients, we report elevated ratios of plasma ApoCIII glycoisoforms to ApoCII in patients who later developed severe preeclampsia. This modulation occurred early in pregnancy (8-12 weeks' gestation), well before onset of clinical symptoms.

Differences in cholesterol, hepatic function, and hemoglobin concentration among cohorts cannot account for our findings. All spectra were normalized to total ion current to correct for possible changes in total protein content originating from hematocrit or blood volume differences among women. Although obesity is a known risk factor for preeclampsia,<sup>3</sup> there was not a statistically significant difference in BMI among our cohorts. Although data suggest that African American heritage elevates risk of preeclampsia, ethnicity was probably not a confounding variable since ethnic background was balanced among cohorts.

Recent studies have shown that plasma from third trimester women with preeclampsia increases monocyte adhesion to endothelial cells.<sup>18</sup> To our knowledge, our study is the first to document a comparable increase in monocyte adhesion before the onset of clinical symptoms. These results are consistent with speculations that the maternal immune response is



**Figure 6. Ratios of ApoCIII to ApoCII predict severe preeclampsia in the first trimester.** Ratios of ApoCIII<sub>1</sub> (at 9.4 kDa) to ApoCII (at 8.2 kDa) (left) and ApoCIII<sub>2</sub> (at 9.7 kDa) to ApoCII (right) were measured by SELDI-TOF MS spectral amplitudes in maternal plasma from gestational hypertensives (n = 10), mild preeclampsics (n = 8), severe preeclampsics (n = 5) or gestationally age-matched normotensives (8-12 weeks, n = 11). \*P < .05 by ANOVA and post hoc analysis with Bonferroni correction. ApoCIII indicates apolipoprotein CIII; SELDI-TOF MS, surface-enhanced laser desorption ionization time of flight mass spectrometry; ANOVA, analysis of variance.

heightened in women with preeclampsia early in pregnancy, around the time of placentation and disease pathogenesis.<sup>3</sup> Further, our results are analogous to comparable studies in the atherosclerosis literature reporting an increase in monocyte adhesion in patients who later developed cardiovascular disease.<sup>6,22</sup> In these studies, ApoCIII was implicated in direct activation and adhesion of monocytes by inducing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) expression and β-integrin expression, respectively.<sup>9</sup> It is conceivable that the elevated ratio of ApoCIII to ApoCII reported in our study contributes causally to the dyslipidemia of preeclampsia, although this hypothesis remains to be directly tested.

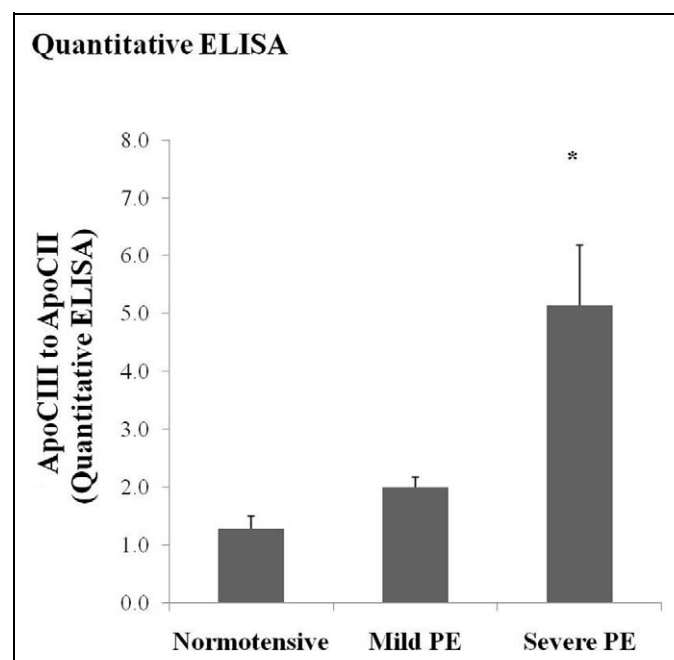
Our study is the first to prospectively evaluate the ratio of ApoCIII glyco-isoforms to ApoCII in asymptomatic women with preeclampsia in the first trimester using the SELDI-TOF MS technology. Our quantitative findings complement a recent proteomic analysis demonstrating a 1.9-fold decrease in ApoC-III in second trimester plasma relative to normotensive controls with concomitant elevations in other proteins associated with atherogenesis.<sup>23</sup> Our quantitative data also suggest a more marked decrease in abundance of ApoCII than ApoCIII by both SELDI-TOF MS and ELISA. The resulting increase in the ratio of ApoCIII glyco-isoforms to ApoCII was particularly pronounced in severe cases of preeclampsia.

The physiological implications of the elevated the ratio of ApoCIII to ApoCII observed in our population of nulliparas are unclear. Since ApoCII functionally opposes ApoCIII, 1 plausible explanation is that the increased ratio of ApoCIII

to ApoCII represents a functional increase in ApoCIII.<sup>24</sup> Whereas ApoCII facilitates lipolysis of very-low-density lipoprotein (VLDL) triglycerides, ApoCIII slows the kinetics of lipolysis reactions by noncompetitively inhibiting ApoCII.<sup>24</sup> VLDL-ApoCIII elevates expression of key inflammatory mediators (eg, NF-κB and β-integrin), promoting leukocyte activation and recruitment to sites of atherogenesis by endothelial expression of adhesion molecules.<sup>9</sup> Therefore, the net effect of the elevated ratio of ApoCIII to ApoCII could be a more pronounced physiological effect of VLDL-ApoCIII in preeclamptic plasma.<sup>25</sup>

Apolipoprotein CIII regulation of lipid homeostasis is not limited to triglyceride-rich proteins. Kinetic analyses demonstrate ready association of ApoCIII with HDL (high-density lipoprotein). After lipoprotein lipase hydrolysis of VLDL triglyceride, VLDL-associated ApoCIII rapidly converts to association with HDL in vitro, although the exact mechanism of conversion remains unknown.<sup>26,27</sup> Sequestration of ApoC-III<sub>2</sub> and/or ApoCII by HDL in plasma is a plausible mechanism to explain the decrease in relative abundance of these apolipoproteins in our preeclamptic cohorts.

Recent evidence suggests that the ratio of ApoCIII<sub>1</sub> to ApoC-III<sub>2</sub> is prognostic for graft versus host disease, liver disease, and obesity.<sup>11</sup> Although these authors did not find the ratio of ApoCIII<sub>1</sub> to ApoCIII<sub>2</sub> to be predictive of preeclampsia, they did not discriminate between mild and severe cases. Many physicians and authors classify mild and severe preeclampsia as 2 discrete syndromes with different pathogenesis, presentation, and



**Figure 7. The Increased ratio of ApoCIII to ApoCII in pre-eclamptic maternal plasma by quantitative competitive ELISA.** ApoCIII and ApoCII were quantified in maternal plasma segregated by diagnostic cohort in competitive ELISA assays using monoclonal antibodies for ApoCIII and ApoCII, respectively ( $n = 5$  patients per cohort). Results are reported as the ratio of ApoCIII to ApoCII (\* $P < .05$  by and post hoc analysis with Bonferroni correction). ApoCIII indicates apolipoprotein CIII; ELISA, enzyme-linked immunosorbent assay.

obstetric outcome.<sup>2</sup> Our cohorts contain an admittedly small sampling of patients ( $n = 5$ -8 patients in the preeclamptic cohorts). However, when severe cases of preeclampsia were segregated from mild cases, we observed a more significant elevation in the ratio of ApoCIII to ApoCII in the severe cases. Gestational hypertensive individuals were not statistically different from normotensive individuals, suggesting that the elevated ratios are unique to preeclampsia and may have predictive value in our nulliparous patient population.

## Conclusion

In conclusion, our study sought to correlate the ratio of ApoCIII glyco-isoforms to ApoCII in maternal plasma from preeclamptic, gestational hypertensive, and normotensive cohorts. In our patient population, our results support the ratios of ApoCIII<sub>2</sub> and ApoCIII<sub>1</sub> to ApoCII as a possible novel biomarker for pre-clinical diagnosis of severe preeclampsia and early differentiation between mild and severe cases.

## Authors' Note

The views expressed in this presentation are those of the authors and do not reflect the official policy of the Department of the Army, the Department of Defense or the US Government.

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## References

1. Widmer M, Villar J, Benigni A, Conde-Agudelo A, Karumanchi SA, Lindheimer M. Mapping the theories of preeclampsia and the role of angiogenic factors: a systematic review. *Obstet Gynecol*. 2007;109(1):168-180.
2. Roberts J. Preeclampsia a two-stage disorder: what is the linkage? Are there directed fetal/placental signals? In: Lyall F, Belfort M, eds. *Preeclampsia: Etiology and Clinical Practice*. Cambridge, UK: Cambridge University Press; 2007:183-194.
3. Hubel C. Dyslipidemia and preeclampsia. In: Lyall F, Belfort M, eds. *Preeclampsia: Etiology and Clinical Practice*. Cambridge, UK: Cambridge University Press; 2007:164-178.
4. Noori M, Savvidou M, Williams D. Endothelial factors. In: Lyall F, Belfort M, eds. *Preeclampsia: Etiology and Clinical Practice*. Cambridge, UK: Cambridge University Press; 2007:50-78.
5. Pijnenborg R, Vercruysse L, Hanssens M, Van Assche FA. Trophoblast invasion in preeclampsia and other pregnancy disorders. In: Lyall F, Belfort M, eds. *Preeclampsia: Etiology and Clinical Practice*. Cambridge, UK: Cambridge University Press; 2007: 1-19.
6. Kawakami A, Aikawa M, Libby P, Alcaide P, Luscinskas FW, Sacks FM. Apolipoprotein CIII in apolipoprotein B lipoproteins enhances the adhesion of human monocytic cells to endothelial cells. *Circulation*. 2006;113(5):691-700.
7. Wang CS, Hartsuck JA, Weiser D. Kinetics of acylglycerol hydrolysis by human milk lipoprotein lipase. *Biochim Biophys Acta*. 1985;837(2):111-118.
8. Chan DC, Chen MM, Ooi EM, Watts GF. An ABC of apolipoprotein C-III: a clinically useful new cardiovascular risk factor? *Int J Clin Pract*. 2008;62(5):799-809.
9. Kawakami A, Osaka M, Aikawa M, et al. Toll-like receptor 2 mediates apolipoprotein CIII-induced monocyte activation. *Circ Res*. 2008;103(12):1402-1409.
10. Bondarenko PV, Cockrill SL, Watkins LK, Cruzado ID, Macfarlane RD. Mass spectral study of polymorphism of the

- apolipoproteins of very low density lipoprotein. *J Lipid Res.* 1999;40(3):543-555.
11. Harvey SB, Zhang Y, Wilson-Grady J, et al. O-glycoside biomarker of apolipoprotein C3: responsiveness to obesity, bariatric surgery, and therapy with metformin, to chronic or severe liver disease and to mortality in severe sepsis and graft vs host disease. *J Proteome Res.* 2009;8(2):603-612.
  12. Mazurkiewicz JC, Watts GF, Warburton FG, Slavin BM, Lowy C, Koukkou E. Serum lipids, lipoproteins and apolipoproteins in pregnant non-diabetic patients. *J Clin Pathol.* 1994;47(8):728-731.
  13. Bai H, Liu X, Liu R, Liu Y, Li M, Liu B. [Analysis of serum lipid and apolipoprotein levels in pregnancy-induced hypertension and normotensive pregnant women]. *Hua Xi Yi Ke Da Xue Xue Bao.* 2002;33(1):58-61.
  14. Chalas J, Audibert F, Francoual J, Le Bihan B, Frydman R, Lindenbaum A. Concentrations of apolipoproteins E, C2, and C3 and lipid profile in preeclampsia. *Hypertens Pregnancy.* 2002;21(3):199-204.
  15. Rasanen J, Girsan A, Reddy A, et al. Maternal serum biomarkers of gestational hypertension distinct from preeclampsia. *Am J Obstet Gynecol.* 2008;199(6 suppl A):S6.
  16. American College of Obstetrics and Gynecology. ACOG Practice Bulletin Bariatric Surgery and Pregnancy. ACOG Practice Bulletin, June 2009. *Obstet Gynecol.* 2009;113(6):1405-1413.
  17. American College of Obstetrics and Gynecology. Diagnosis and management of preeclampsia and eclampsia. ACOG Practice Bulletin, January 2002. *Obstet Gynecol.* 2002;99(1):159-167.
  18. Ryu S, Huppmann AR, Sambangi N, Takacs P, Kauma SW. Increased leukocyte adhesion to vascular endothelium in preeclampsia is inhibited by antioxidants. *Am J Obstet Gynecol.* 2007;196(4):400e1-400e7; discussion e7-e8.
  19. Burtis C, Ashwood ER, Tietz NW. *Tietz Textbook of Clinical Chemistry.* Philadelphia, PA: Saunders; 1999.
  20. Cai S, Davis AE III. Complement regulatory protein C1 inhibitor binds to selectins and interferes with endothelial-leukocyte adhesion. *J Immunol.* 2003;171(9):4786-4791.
  21. Nelsestuen GL, Zhang Y, Martinez MB, et al. Plasma protein profiling: unique and stable features of individuals. *Proteomics.* 2005;5(15):4012-4024.
  22. Kawakami A, Yoshida M. Apolipoprotein CIII links dyslipidemia with atherosclerosis. *J Atheroscler Thromb.* 2009;16(1):6-11.
  23. Blumenstein M, McMaster MT, Black MA, et al. A proteomic approach identifies early pregnancy biomarkers for preeclampsia: novel linkages between a predisposition to preeclampsia and cardiovascular disease. *Proteomics.* 2009;9(11):2929-2945.
  24. Huff MW, Fidge NH, Nestel PJ, Billington T, Watson B. Metabolism of C-apolipoproteins: kinetics of C-II, C-III1 and C-III2, and VLDL-apolipoprotein B in normal and hyperlipoproteinemic subjects. *J Lipid Res.* 1981;22(8):1235-1246.
  25. Shachter NS. Apolipoproteins C-I and C-III as important modulators of lipoprotein metabolism. *Curr Opin Lipidol.* 2001;12(3):297-304.
  26. Cohn JS, Rodriguez C, Jacques H, Tremblay M, Davignon J. Storage of human plasma samples leads to alterations in the lipoprotein distribution of apoC-III and apoE. *J Lipid Res.* 2004;45(8):1572-1579.
  27. Ordovas JM, Cassidy DK, Civeira F, Bisgaier CL, Schaefer EJ. Familial apolipoprotein A-I, C-III, and A-IV deficiency and premature atherosclerosis due to deletion of a gene complex on chromosome 11. *J Biol Chem.* 1989;264(28):16339-16342.